



Porous chitosan by crosslinking with tricarboxylic acid and tuneable release

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Abstract

Chitosan hydrogels crosslinked with 1,3,5-benzene tricarboxylic acid (BTC) are readily prepared at room temperature by adding aqueous chitosan solution dropwise into BTC-ethanol solution. Highly interconnected porous chitosan materials are subsequently prepared by freeze-drying the chitosan hydrogels. These chitosan materials show porous structures with smaller pores than conventionally prepared chitosan hydrogels via crosslinking with NaOH, genipin or sodium triphosphate. This method of forming chitosan hydrogels with BTC provides the advantage of facile encapsulation of both hydrophobic and hydrophilic compounds, as demonstrated with the model dyes (Oil Red O and Rhodamine B). The release of the hydrophilic dye from the chitosan hydrogels is demonstrated and can be tuned by BTC/chitosan concentrations and the hydrogel drying methods. However, the release of encapsulated hydrophobic dye is negligible.

Keywords Porous chitosan · Freeze drying · Benzene tricarboxylic acid · Tuneable release · Encapsulation

1 Introduction

Porous polymers and hydrogels have been extensively used as scaffolds for tissue engineering and as carriers for drug delivery [1, 2]. Porous polymers can be generated by suitable drying of hydrogels. These materials can be formed from both synthetic polymers and natural polymers [1]. The basic requirements to generate such materials for biomedical applications include biocompatibility, biodegradability, mild processing conditions, and the ease for further modification. Chitosan is a cationic polymer of natural origin and has been extensively used as biocompatible scaffold or hydrogel for a wide range of biomedical applications, including tissue engineering [3, 4], drug delivery [5–8], and many others [9–12]. Chitosan is the deacetylation product of chitin. Chitin is a natural polysaccharide, poly(β -(1 → 4)-N-acetyl-D-glucosamine), and

is the second most abundant polymer after cellulose [13]. The common sources of chitin are crab and shrimp shells. There are two forms of chitin, α -chitin and β -chitin, with α -chitin being the most common. It is difficult to dissolve chitin in conventional solvents, which limits its applications. Chitin can be deacetylated under heating in concentrated NaOH to produce chitosan [14].

Chitosan is usually available as a copolymer, defined by the degree of deacetylation. When the degree of deacetylation is greater than 50% (The exact number of deacetylation degree may be debatable. Some researchers define chitosan as > 60–70% deacetylation of chitin), it is called ‘chitosan’ and can be dissolved in acidic water [13]. The structure of chitosan (considering it is 100% deacetylated) is very similar to cellulose, with $-\text{NH}_2$ group replacing one of the $-\text{OH}$ groups on cellulose. The presence of the $-\text{NH}_2$ groups on chitosan is significant. It can be protonated to

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dissolve chitosan in water (providing positive charges) and offers facile chemical modifications [13]. One of the most common methods to modify chitosan is by carboxylation at the –O or –N position [13]. Carboxymethyl chitosan is soluble in neutral water and has found unique applications in drug delivery and tissue engineering [15]. There are also other chemical modifications [13], for example, amino acid-modified chitosan for biomedical applications [16]. Chitosan can also be blended with other polymers to produce hybrid hydrogels, *e.g.*, chitosan–alginate hydrogel for tumour treatment [17], collagen–chitosan for endothelial differentiation and angiogenesis [18].

Chitosan hydrogels can be formed by chemical crosslinking, physical crosslinking, or electrostatic interaction [9, 10, 19]. Due to the positive charge of chitosan in solution, multivalent anions or negatively charged polymers (*e.g.*, certain proteins, nucleic acids) can be used to form hydrogels via ionic interaction. Another way to produce physically crosslinked hydrogels is by adding base into a chitosan solution. The increase of pH leads to deprotonation of chitosan, reduced solubility, and self-assembled gels by H bonding, hydrophobic interaction, and Van der Waals forces. This can be simply achieved by adding NaOH or Na₂CO₃. However, these hydrogels are heterogeneous due to the rapid change of local pH. Chitosan hydrogels for cell culturing are usually produced by crosslinking chitosan with calcium phosphate [5], sodium triphosphate [20], and β -glycerol phosphate [21–23]. Chitosan is often chemically crosslinked with glutaraldehyde [13, 24]. However, due to its toxicity, glutaraldehyde can be replaced with biocompatible crosslinkers such as heparin [25] and genipin [26]. Chitosan hydrogels may be prepared as bulky gels (by simple gelation of chitosan solution), injectable gels [21–23], microparticles [27], nanoparticles [14, 28], or nanofibers [29].

Chitosan is usually dissolved in water with the aid of monocarboxylic acid such as acetic acid and formic acid. Dicarboxylic acid such as oxalic acid, succinic acid, malic acid, and adipic acid have been used to dissolve chitosan and also act as crosslinkers to form chitosan hydrogels. The chitosan gels with dicarboxylic acid were freeze dried [30] or air dried [31] that could take up water as hydrogels with improved mechanical strength [30]. However, to form hydrogels without the drying step, a mixture of N-hydroxy-succinimide (NHS) and 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide (EDC) is often required to facilitate the gelation process [32, 33].

Here, we report the preparation of chitosan hydrogels using 1,3,5-benzyl tricarboxylic acid (BTC) as crosslinker by simply dropping aqueous chitosan solution into BTC-ethanol solution. The reaction between the –NH₂ groups on chitosan and the –COOH groups on BTC is the key to the formation of chitosan gel in a water–ethanol mixture.

Porous chitosan can be subsequently produced via a freeze-drying process. This method avoids the use of basic solution and ensure the gel preparation at room temperature. Due to the wide use of chitosan as biocompatible polymers, the porous chitosan materials are further investigated as scaffolds for controlled drug release. Since drug compounds can be hydrophilic or hydrophobic, in order to demonstrate the potential of controlled release, both hydrophilic dye (Rhodamine B) and hydrophobic dye (Oil Red O) are used as model compounds because they can be easily monitored. The fabrication method of porous chitosan by crosslinking with BTC is shown to provide an effective route for the encapsulation of both water-soluble and ethanol-soluble (hydrophobic) active substances (dyes used as models in this study). The controlled release of the hydrophilic compound from the chitosan hydrogel is further demonstrated, whilst the release of hydrophobic dye is negligible. To the best of our knowledge, this is the first report to use BTC as a crosslinker for the preparation of porous chitosan, although there is a report to form supramolecular hydrogels from BTC and hydroxyl pyridines, via the interaction between the –COOH group and –N containing group [34].

2 Experimental

2.1 Chemicals and reagents

Sodium triphosphate pentabasic (NaTPP, 72,061, Sigma-Aldrich), NaOH (795,429, Sigma-Aldrich), chitosan (medium molecular weight, 75–85% deacetylated, 448,877, Sigma Aldrich), acetic acid glacial (A6283, Sigma Aldrich), Rhodamine B (25,243–3, Aldrich), Oil Red O (O0625, Sigma-Aldrich), glutaraldehyde solution (25 wt%) (G6257, Sigma-Aldrich), 1,3,5-benzyl tricarboxylic acid (BTC, 482,749, Sigma Aldrich), and hydrochloric acid (1 N, H9892, Sigma Aldrich) were used as purchased. Genipin was purchased from Alpha Laboratories Ltd. Distilled water was used for all the water solutions.

2.2 Preparation of chitosan hydrogels and porous chitosan

Chitosan hydrogels were termed as ‘A-BB’ where the first number ‘A’ means the weight concentration (wt%) of chitosan solution in water and the second number ‘BB’ indicates the molar concentration (mM) of BTC solution in ethanol. For example, chitosan hydrogel 1–50 means the hydrogel prepared from 1 wt% chitosan solution and 50 mM BTC-ethanol solution. To prepare chitosan hydrogel 1–50, chitosan solution (1 wt%) was prepared by dissolving chitosan (medium molecular weight, 2.50 g) in 250 mL

0.6 v/v% acetic acid solution with stirring and mild heating at 40 °C overnight. BTC solution (50 mM) was prepared by dissolving BTC powder (2.625 g, 12.5 mmol) in 250 mL ethanol. A 5 mL glass syringe with a 25 gauge stainless steel needle was used to inject 1.0 mL chitosan solution into 10.0 mL BTC solution drop by drop in a test tube. Hydrogel beads could be formed instantly (concentration dependent) while chitosan drops were sinking into the BTC solution with sufficient mechanical strength to maintain the bead shape. The gel beads were kept in the solution for at least 12 h before filtration and washing with distilled water (5 mL \times 3). Similar procedures were used to prepare chitosan hydrogels 1–10 and 0.5–50 using different concentrations of chitosan solutions and BTC solutions.

For the preparation of chitosan hydrogel film on coverslip, 50 μ L 1 wt% chitosan was pipetted on a 13 mm coverslip and evenly spread and then soaked into the BTC (50 mM) solution. Gel dots on coverslip were prepared by placing chitosan solution droplets on coverslip instead of evenly spreading chitosan solution before soaking into the BTC solution.

For the preparation of conventional chitosan hydrogel beads or dots on coverslip, 1 wt% chitosan solution was processed with 10 w/v% NaTPP aqueous solution (10 g NaTPP in 100 mL water) or 0.1 M NaOH aqueous solution, using the same procedure as above. Because the chitosan hydrogel prepared from 0.1 M NaOH solution was very weak, it was further treated by soaking in 0.5 w/v% genipin solution (0.1 g genipin dissolved in 20 mL 1:1 ethanol–water by volume) at room temperature for 1 h.

Porous chitosan was prepared after freezing the as-prepared chitosan hydrogels followed by a freeze-drying process (48 h) using a CoolSafe freeze dryer from Jencons-VWR.

2.3 Encapsulation of hydrophilic/hydrophobic dyes in chitosan hydrogels

Oil Red O (OR) was used as a hydrophobic dye. OR (0.125 g) was dissolved in 250 mL of BTC solution to prepare 0.05 w/v% OR-BTC solution. The same hydrogel preparation procedure, as described in 2.2, was applied to produce OR loaded 1–50, 1–10 and 0.5–50 hydrogels.

Rhodamine B (RB) is soluble in both water and ethanol and was used as a hydrophilic dye. RB (0.05 g) was dissolved in 50 mL chitosan solution of different concentrations to prepare 0.1 w/v% RB-chitosan solution. The same hydrogel preparation procedure was again used to prepare RB loaded 1–50, 1–10 and 0.5–50 hydrogels.

Both dyes were primarily physically trapped within the chitosan hydrogels. There were other interactions involved, including hydrogen bonding between Rhodamine B and

chitosan and possible π – π interactions between both dyes and BTC.

2.4 Determination of loading capacity and encapsulation efficiency

RB loaded hydrogel samples were dissolved in 3 mL HCl (0.1 M) solution and then diluted in a 10 mL volumetric flask with distilled water. OR-loaded hydrogel samples were dissolved with 7 mL HCl (0.1 M) solution and then diluted in a 10 mL volumetric flask with distilled water. The UV/Vis analysis of each dissolved hydrogel solution was performed, and the concentration of the dye in the solution was calculated by the calibration curves (Fig. S1 and Fig. S2). The encapsulation efficiency (E) and loading efficiency (L) were calculated by the equations below, respectively:

$$E = M_{ED}/M_{TD}$$

$$L = M_{ED}/M_C$$

where M_{ED} is the mass of encapsulated dye, M_{TD} is the total mass of dye used, M_C is the mass of chitosan used in the preparation.

2.5 Release of the dyes from chitosan hydrogels

2.5.1 Preparation of phosphate buffer saline (PBS) solution

KH_2PO_4 (0.61 g, 4.5 mmol), NaH_2PO_4 (0.088 g, 7.3 mmol), NaOH (0.4 g, 10 mmol), and NaCl (8.17 g, 139.7 mmol) were dissolved in 1 L distilled water. HCl solution (0.1 M) was carefully added with stirring while pH of the solution was monitored by a pH meter until pH 7.4 was achieved.

2.5.2 RB release study

RB-loaded hydrogel samples (prepared from 1 mL 1 wt% chitosan solution) were immersed into 50 mL PBS solution in a beaker with gentle stirring at 37 °C. At intervals of 30 s, 300 μ L of solution was pipetted from the solution for UV–Vis measurement, and 300 μ L of fresh PBS solution was added back to the beaker. The interval was gradually increased to 1 min, 2 min, 5 min and 10 min after 5 min, 10 min, 20 min and 30 min of release, respectively. The release study was monitored for up to 120 min. This method was applied to determine the release profile of 1–50, 1–10 and 0.5–50 RB-loaded hydrogel samples when they were wet, air dried and freeze dried.

The same procedure was applied to evaluate the release behaviour of OR-loaded hydrogels.

2.6 Characterizations

The morphology of the materials was observed by a Hitachi-S4800 scanning electron microscope (SEM). A small piece of the material was adhered to a stud using double-sided carbon tape. Dry hydrogel samples were coated with gold using a sputter-coater (EMITECH K550X) for 2 min at 25 mA before SEM imaging. An Olympus CX41 microscope with CellSens entry imaging software was used to image hydrogel beads. The viscosity of chitosan solution was measured using a plate rheometer (ARES-G2, TA Instrument). The measurement was carried out with a 50.0 mm 1 degree stainless steel cone plate at a temperature of 25 °C and a soak time of 60 s in a shear rate range of 0.1–500 1/s. ^1H NMR Analysis was done using the Bruker Fourier 300HD with D₂O as solvent. The Brunauer–Emmett–Teller (BET) surface area by N₂ sorption at 77 K was measured using a Micromeritics ASAP 2420 adsorption analyzer. Powder X-ray diffraction (PXRD) patterns were collected on a Bruker-AXS D8 advanced diffractometer with CuK radiation source. Fourier transform infrared (FTIR) spectra were collected using a Perkin-Elmer 457 spectrometer (PerkinElmer, Buckinghamshire, UK). No internal standard was added for the FTIR analysis because the chitosan gels were crosslinked and it was impossible to add an internal standard uniformly. The FTIR data were uploaded, overlapped, and normalized using the OPUS Software. Elemental analysis was performed using a Thermo flash EA 112 series instrument. For the release study by UV–Vis spectroscopy, a UV plate reader (μ Quant, Bio-Tek Instruments, Inc.) with an acrylic 96-well plate was used to measure the concentrations of RB or OR released into the PBS solution.

3 Results and discussion

3.1 Characterization and morphology of chitosan and crosslinked porous chitosan

The deacetylation degree and molecular weight of chitosan are very important parameters. In this study, chitosan of medium molecular weight was obtained from Sigma Aldrich. The element analysis of the chitosan showed the content of N at 7.60%. Based on the calculations (N contents of 6.90% for chitin and 7.73% for 100% deacetylated chitosan), this chitosan exhibited a deacetylation degree of 84%. ^1H NMR analysis of the chitosan solution in D₂O with a small amount of HCOOH was carried out (Fig. S3). The integration area of the signal (1.97 ppm, 3H) from the acetyl content in reference to the D-glucosamine residue (6H) gave rise to a deacetylation degree of 88%, which is reasonably consistent with the result from

microanalysis. It was found that the viscosity of 1 wt % chitosan solution in water with 0.6 v/v% acetic acid was 0.16 Pa S at the shear rate of 10 1/s. The viscosity decreased generally with the increasing shear rate, as shown in Fig. S4. When preparing crosslinked chitosan, BTC was dissolved in ethanol and used as the crosslinking reagent. The low density of ethanol solution allows the drops of aqueous chitosan solution sink through. When injecting chitosan solution using a syringe, the droplets could form gel beads in the BTC solution before hitting the bottom. This was dependent on the rate of gelation, determined by the concentrations of chitosan and BTC. Higher concentration resulted in fast gelation, which could produce chitosan gel beads with sufficient mechanical stability so that no deformation of the beads occurred when precipitating to the bottom of the glass beaker. The gel beads were kept in the BTC solution for 12 h at room temperature, to allow for full gelation.

As shown in Fig. 1a, stable hydrogel beads were formed when 1wt% chitosan and 50 mM BTC solutions were employed. The floating of beads was due to the air trapped inside during injection and fast gelation in BTC ethanol solution. When the BTC concentration or chitosan concentration was decreased, the bead shape was lost. Instead, a much swollen hydrogel (Fig. 1b) and a fiber-like hydrogel (Fig. 1c) were generated, respectively. For the samples shown in Fig. 1, OR-BTC solutions were used to form chitosan hydrogels. The red gels, particularly, the red gel beads, demonstrate the successful encapsulation of OR in chitosan hydrogels.

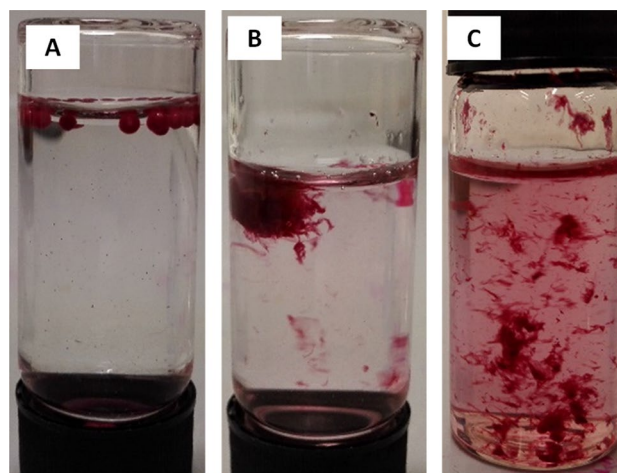


Fig. 1 Images of chitosan hydrogels prepared by injecting aqueous chitosan solution into BTC-ethanol solution containing 0.05 w/v% Oil Red O: **a** chitosan hydrogel 1–50, **b** chitosan hydrogel 1–10, **c** chitosan hydrogel 0.5–50 dyed. After completing the injection, the gels were filtered, rinsed with distilled water 3 times, and then transferred into glass vials with water

The chitosan hydrogel 1–50 beads were mechanically stable and could be handled with ease. These beads could be air dried with small shrinkage. Figure 2a shows a SEM image of the dry beads. The surface structure is dense, with small mesopores observed under higher magnification (Fig. 2b). Chitosan hydrogels 1–10 and 0.5–50 could also be dried, but with significant shrinkage. Similar, very dense porous structures were observed (Fig. 2c, d).

Freeze-drying is a process that can be used to produce highly porous materials with minimal shrinkage with or without ice crystals as templates [35]. When chitosan hydrogel 1–50 beads were freeze-dried, no shrinkage was observed. Also, like the air-dried beads, the freeze-dried beads showed wrinkled surface (Fig. S5), which was attributed to the freezing of gels beads using liquid nitrogen before freeze drying. Although the surface of the freeze dried beads was more porous than that of the air-dried beads, a dense surface was still the feature of these freeze-dried beads (Fig. S5).

However, the internal structure of the freeze-dried chitosan hydrogel is highly porous, as revealed by imaging the sectioned surface. Highly interconnected porous structure can be seen for all these freeze-dried chitosan hydrogels by SEM imaging (Fig. 3). The pore structure for freeze-dried chitosan 1–50 is similar, with only BTC (Fig.

S6) or with loaded OR (Fig. 3a, b). The pore structure is dense and the pore sizes are in the region of mesopores (~ 50 nm). However, with the loaded OR, there appear many nanoparticles within the porous structure (Fig. 3b). Because OR is hydrophobic and it cannot be molecularly mixed with the hydrophilic chitosan, those bright spots are most likely to be OR nanoparticles (Fig. 3b). With the decrease of BTC concentration (chitosan hydrogel 1–10, Fig. 3c, d) or chitosan concentration (chitosan hydrogel 0.5–50, Fig. 3e, f), both of the freeze-dried chitosans show highly interconnected macropores, around 50–200 nm and quite uniformly around 50–100 nm, respectively.

The freeze-dried chitosan beads were also characterized by other techniques. Based on the PXRD analysis, the beads generated were amorphous, consistent with the previous finding of freeze-dried chitosan [36]. While the macroporous structure was revealed by SEM imaging (Fig. 3), N_2 sorption analysis for micropores/mesopores was performed on the 1–50 chitosan beads, which gave a very low surface area of $12 \text{ m}^2/\text{g}$, indicating the lack of mesopores and micropores. FTIR analysis was carried out on as-purchased chitosan powder, 1–10, and 1–50 beads, as shown in Fig. 4. The characteristic peaks of chitosan is clearly shown [37] while the strong peaks in the region of $1000\text{--}1750 \text{ cm}^{-1}$ show the presence of BTC in the

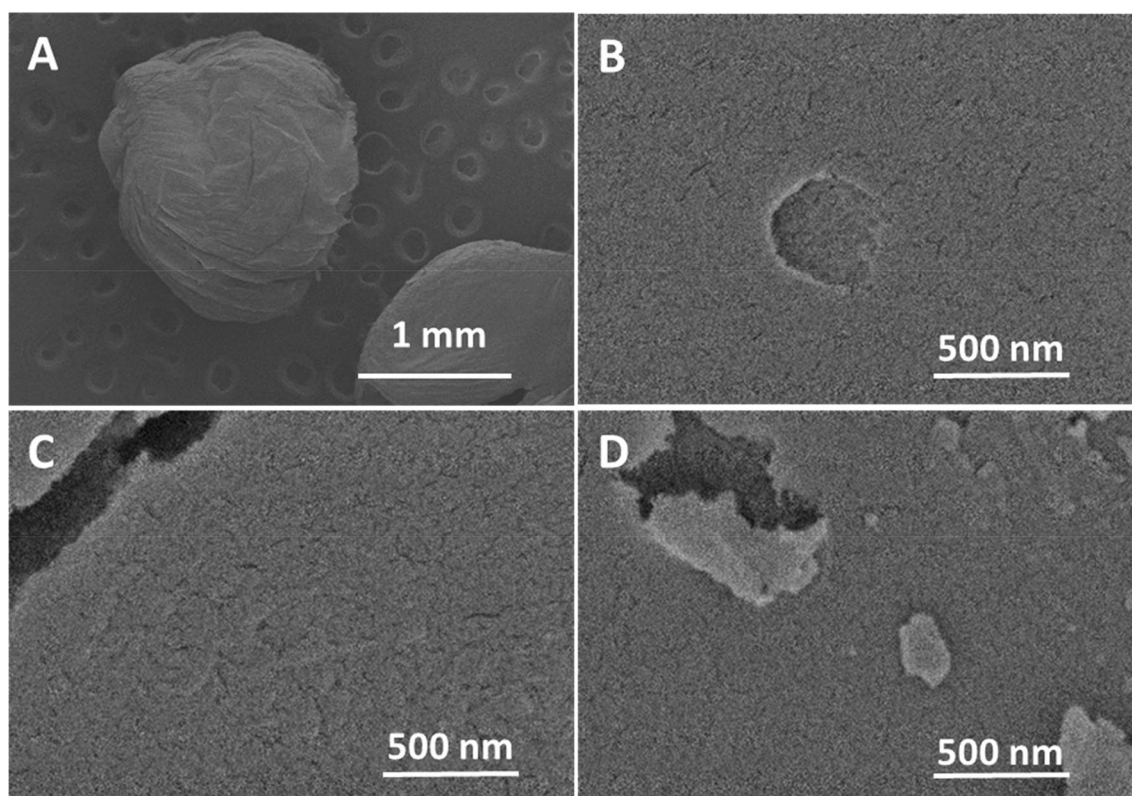


Fig. 2 SEM images of air dried BTC-chitosan hydrogels. **a, b** Chitosan hydrogel 1–50, **c** chitosan hydrogel 1–10, **d** chitosan hydrogel 0.5–50

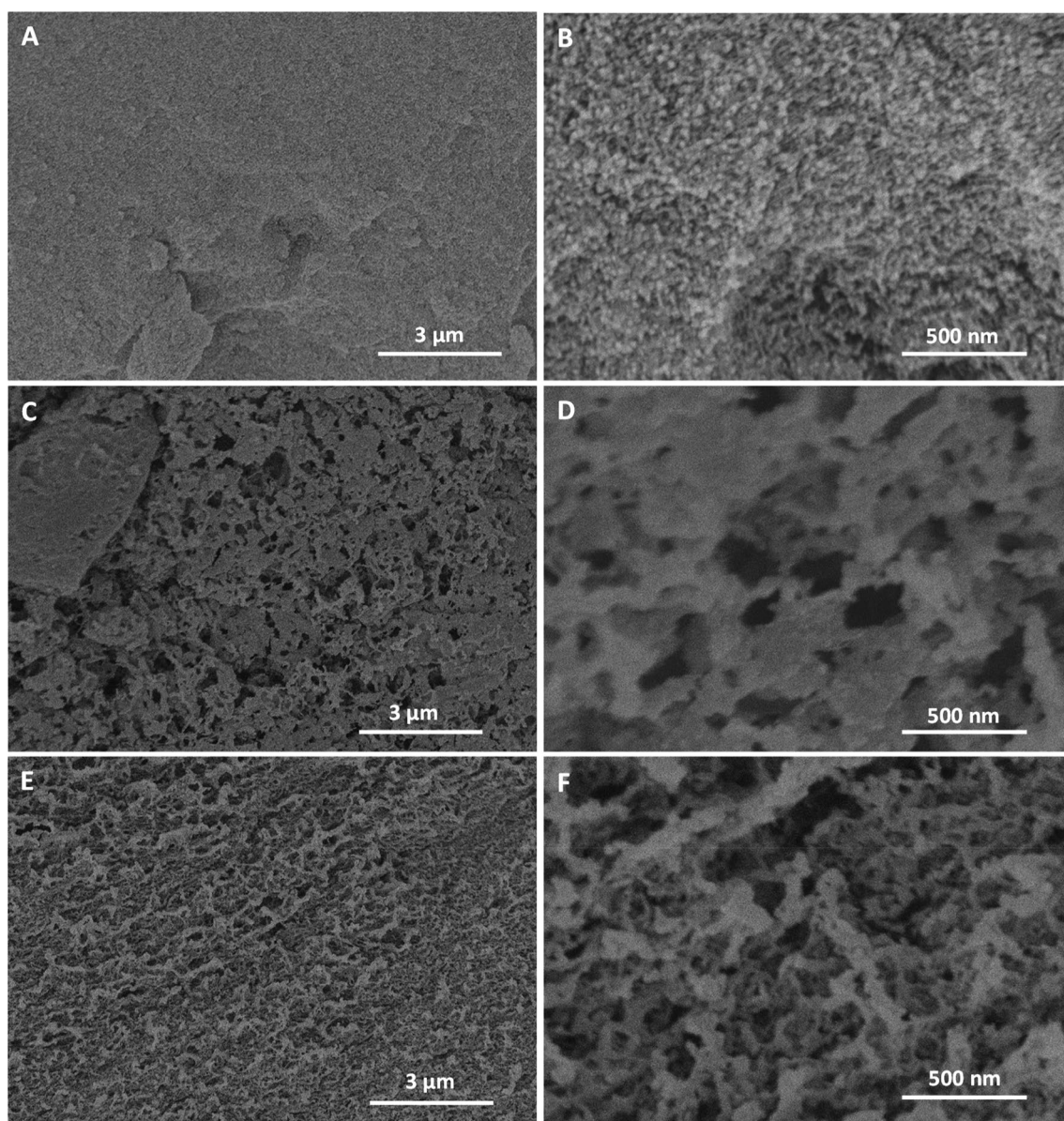


Fig. 3 SEM images of OR-loaded chitosan hydrogel 1–50 (**a, b**), chitosan hydrogel 1–10 (**c, d**), and chitosan hydrogel 0.5–50 (**e, f**)

crosslinked 1–10 and 1–50 chitosan beads. It is difficult to work out the composition of BTC from the FTIR spectra because of the overlapping peaks between chitosan and BTC. Based on the mass increase (the difference between the crosslinked beads and the beads prepared by directly freeze-drying chitosan solution), it was estimated that 3% BTC and 21% BTC (compared to chitosan by mass ratio) were present in 1–10 and 1–50 crosslinked beads.

As comparison, chitosan beads were also prepared using the conventional reagents sodium triphosphate (NaTPP) and NaOH to induce gelation. In the preparation, aqueous chitosan solution was added dropwise in the aqueous solution containing NaTPP or NaOH. Under

similar preparation conditions, the gel beads formed from NaOH and NaTPP were much weaker. Due to the poor mechanical stability, the chitosan hydrogel beads formed from NaOH solution were further chemically crosslinked using the biocompatible crosslinker genipin. Compared to the porous chitosan conventionally formed with NaOH/genipin or NaTPP (Fig. 5), the pore size of the porous chitosan crosslinked with BTC (50–200 nm, Fig. 3) is much smaller. As shown in Fig. 5, the pore sizes of porous chitosan formed with NaOH/genipin are in the region of 1–3 μm whilst the pore sizes are in the region of 1–5 μm for porous chitosan crosslinked with NaTPP.

Fig. 4 FTIR spectra of as-purchased chitosan (blank line), 1–10 crosslinked chitosan (green line), and 1–50 crosslinked chitosan (blue line)

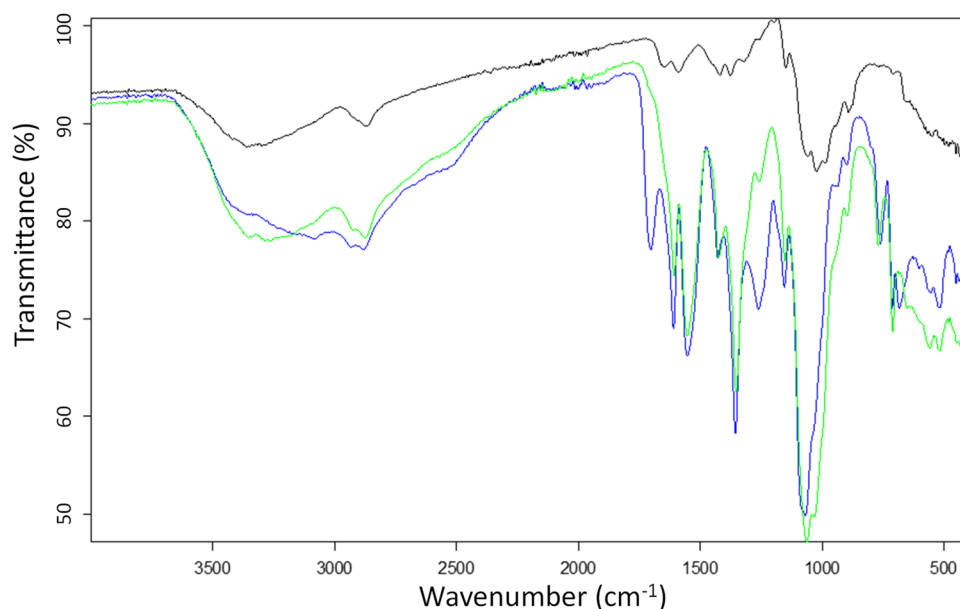
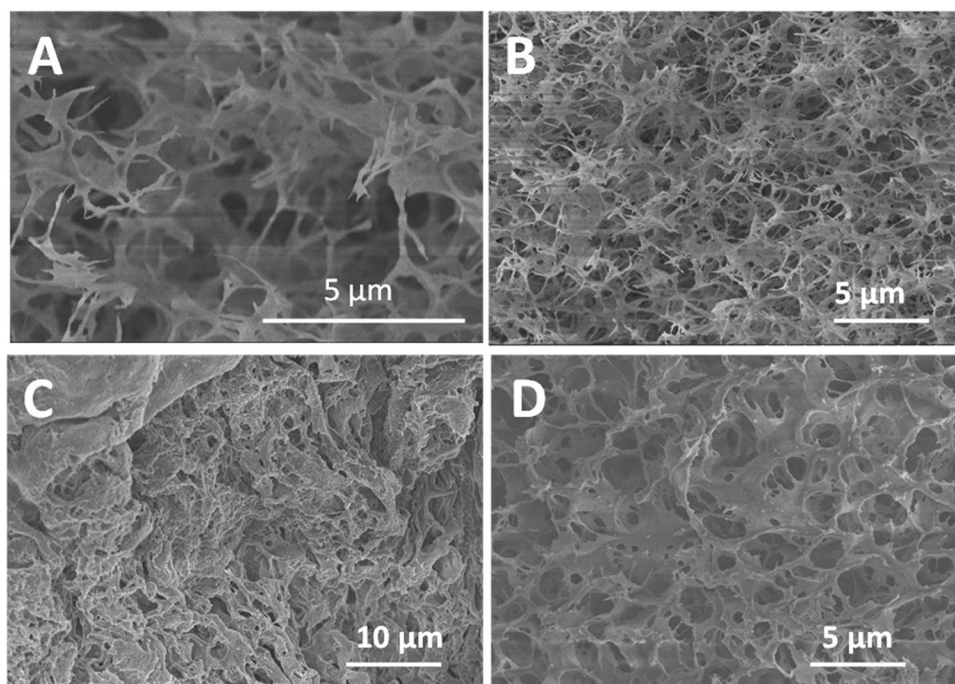


Fig. 5 The interconnected macroporous structures of the freeze-dried chitosan beads prepared from 1 wt% chitosan solution with NaOH/genipin and NaTPP as crosslinkers. **a**, **b** Chitosan beads were firstly formed in dilute NaOH solution and then crosslinked by genipin; **c**, **d** Chitosan beads were formed directly by dropping chitosan solution into the aqueous NaTPP solution



This BTC gelation method is simple, effective and convenient because the gelation occurs readily at room temperature in one step. Compounds containing two carboxylic groups (*e.g.*, glutamic acid, succinic acid) were used to crosslink chitosan or enhance chitosan gel properties. However, either heating the solution (*e.g.*, 70 °C) [32] or an additional room temperature crosslinking mechanism is required [33]. Due to the simplicity of our method, chitosan gel beads, dots, and films could be readily prepared. For example, chitosan gel film could be

prepared by evenly spreading chitosan solution onto a coverslip and then soaked into BTC solution. Hydrogel dots on coverslip were prepared by placing chitosan solution droplets on coverslip and then immersed into the BTC solution.

3.2 Encapsulation of hydrophilic/hydrophobic dyes

Encapsulation can be achieved via three mechanisms, including diffusion, entrapment, and tethering [19]. In

Table 1 Encapsulation of RB and OR into the BTC-crosslinked chitosan hydrogels

Chitosan hydrogel	Chitosan concentration (wt%)	BTC concentration (mM)	Dye	Encapsulation efficiency (%)	Loading efficiency (%) ^a
1–50	1	50	RB	35	0.30
1–10	1	10	RB	34	0.35
0.5–50	0.5	50	RB	9	–
1–50	1	50	OR	3.2	0.96
1–10	1	10	OR	3.5	1.53
0.5–50	0.5	50	OR	2	–

^aLoading efficiency for 0.5–50 hydrogels could not be obtained due to the fragile pieces of hydrogels formed

this study, RB was mainly loaded by entrapment with diffusion-induced leaking whilst OR was completely loaded by diffusion. Table 1 shows the encapsulation efficiency of BTC-chitosan hydrogels towards RB and OR under different conditions. The encapsulations of both OR and RB were achieved by all chitosan hydrogels prepared in this study. Notably, the encapsulation efficiency of RB is much higher than that of OR. For the encapsulation of the same dye, the use of higher concentration of chitosan or BTC solutions generally leads to higher encapsulation efficiency. The loading efficiency of the dyes was also calculated and given in Table 1. Because the dyes were used as indicating model compounds and the dye solutions of very low concentrations were used in the preparation process, the loading capacities of dyes within chitosan hydrogels were very low.

When encapsulating RB into chitosan hydrogels, RB was firstly dissolved in aqueous chitosan solution, which was then injected into BTC-ethanol solution. In theory, the encapsulation efficiency of RB in chitosan hydrogel would be 100%. The lower efficiency in Table 1 is due to the diffusion of RB from chitosan hydrogel into the surrounding medium. The short diffusion path in the fiber-like chitosan hydrogel 0.5–50 leads to the lowest efficiency. The interconnected porosity also plays an important role in enhancing the diffusion of RB from the hydrogel. To improve encapsulation efficiency, higher concentrations of chitosan solution and BTC solutions should be used, resulting in denser hydrogels and slower diffusion. In addition, beads aging time, washing time and the number of washings should also be limited.

The encapsulation of OR is due to the diffusion into the hydrogel. This is accompanied with the diffusion of BTC to chitosan and subsequent crosslinking reaction. This diffusion is limited by equilibrium distribution of OR in the hydrogel and the ethanol solution. The very low efficiency shown in Table 1 is also attributed to the small volume ratio of chitosan solution to OR-BTC solution (1 mL:10 mL). Increasing the volume ratio of chitosan solution to BTC solution may improve the encapsulation efficiency while

increasing the dye concentration in the BTC solution should increase the amount of OR encapsulated in chitosan hydrogel.

3.3 Release of encapsulated dyes from porous chitosan

Controlled release and delivery is one of the main applications for biocompatible hydrogels or porous structures. The release of encapsulated RB and OR from BTC-crosslinked hydrogels was thus investigated. As shown in Figs. 6 and 7, the data points are quite scattered, a simple exponential fit ($P = A(1 - \exp(-kt))$, where A and k are fitting parameters, t is release time, and P is the release percentage of the dye), was used to show the release trend.

Figure 6 shows the cumulative release profiles of RB from chitosan hydrogel 1–50. The hydrogels were assessed as prepared and also freeze-dried or air-dried

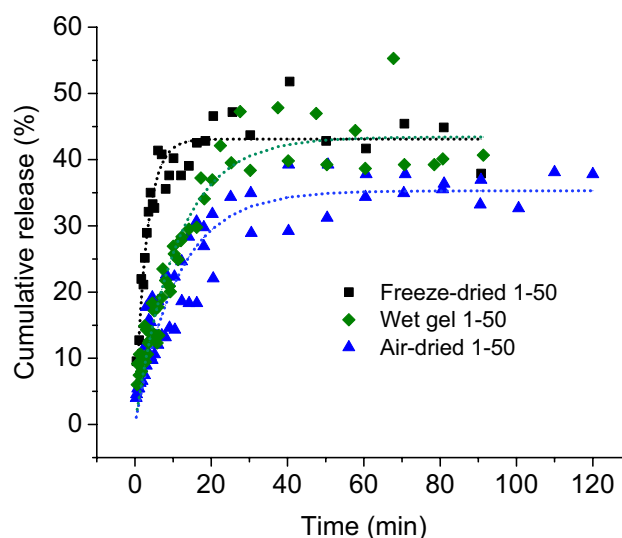


Fig. 6 The release profiles of RB from 1–50 BTC-chitosan hydrogels under different drying conditions: freeze-dried, air-dried, and as-prepared wet hydrogel

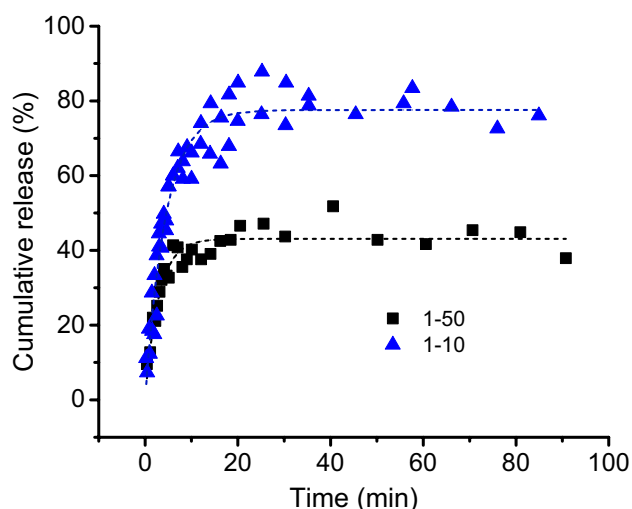


Fig. 7 The release profiles of RB from freeze-dried 1–50 and 1–10 BTC-chitosan hydrogels

before the release study in the PBS solution. A fast release was observed for all the samples, reaching the equilibrium concentration within 1 h. The gels beads were left in the PBS solution for 1 day but no noticeable further release was observed. This fast release is attributed to the highly interconnected pores present in the chitosan materials. As shown in Fig. 6, RB is released faster from the freeze-dried hydrogel than from as-prepared wet hydrogel. This is because the freeze-drying process creates non-shrunk and highly interconnected porous structure. However, for the air-dried chitosan hydrogel, due to the shrinkage during drying and the resulting dense structure, the diffusion into and out of the hydrogel is hindered. A slower release than that of wet chitosan hydrogel is thus obtained.

In order to investigate the effect of hydrogels prepared from varied concentrations of chitosan and BTC solutions, the release behaviour of RB from freeze-dried chitosan hydrogels of 1–50 and 1–10 was investigated. The freeze-dried chitosan hydrogel 1–10 showed larger pores and higher porosity than chitosan hydrogel 1–50 (Fig. 3). It is not surprising that a faster release has been observed from the freeze-dried chitosan hydrogel 1–10 (Fig. 7). RB-loaded hydrogel 0.5–50 was not assessed for release because it was mechanically too weak to be handled with.

The release of OR from similarly loaded chitosan hydrogels was also evaluated. However, due to the poor solubility of OR, there was no detected release of OR from all these samples, as measured by UV–Vis spectroscopy, even when the soaking time was extended

to 3 days. As nanoformulation is known to increase the solubility of poorly water-soluble drugs [38], the BTC-crosslinked chitosan hydrogel may be used to encapsulate hydrophobic drugs in future study.

4 Conclusion

A new method of preparing porous chitosan/chitosan hydrogels using 1,3,5-benzene tricarboxylic acid (BTC) as a crosslinker has been demonstrated. The hydrogels can be readily formed at room temperature simply by dropping aqueous chitosan solution into the ethanol-BTC solution. The gel beads are formed instantly with suitable BTC and chitosan concentrations. Chitosan hydrogel dots or films may be similarly formed by spreading or dotting the chitosan solution on a glass coverslip which is then immersed in the ethanol-BTC solution. Highly porous chitosan materials are generated by freeze-drying the prepared chitosan hydrogels. The pore size and pore network of the porous chitosan formed with BTC is much smaller than the porous chitosan conventionally prepared with NaOH/genipin and sodium triphosphate. Using the BTC-crosslinking method, both hydrophilic dyes and hydrophobic dyes can be encapsulated into chitosan hydrogels. Tuneable release of the hydrophilic dye (Rhodamine B) from porous chitosan and chitosan hydrogels is further demonstrated, although the release of hydrophobic dye (Oil Red O) is highly limited. This method provides an effective way for preparation of chitosan hydrogels/porous chitosan and encapsulating potential hydrophilic drug compounds for the controlled release.

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Compliance with ethical standards

Conflict of interest On behalf of all authors, the corresponding author states that there is no conflict of interest.

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